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β-Adrenergic Receptors during Development

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Introduction

The specificity of the response of pineal glands to stimulation with β-adrenergic agonists and the blockade of response by adrenergic antagonists provided pharmacologic evidence of the presence of β-adrenergic receptors in this tissue prior to the availability of radiolabelled binding ligands which could bind specifically to the receptors [10, 19]. Of particular interest were studies which demonstrated that following stimulation with norepinephrine, adenylate cyclase was activated, cyclic AMP accumulated, and new protein synthesis was triggered which led to the induction of serotonin *N*-acetyltransferase activity (fig. 1) [10]. This in turn resulted in increased melatonin production. With this understanding of the regulation of *N*-acetyltransferase activity it was logical to ask at what point in development pineal tissue became responsive to adrenergic stimulation and thus, implicitly, when do β-adrenergic receptors appear. In a study of developmental aspects of adrenergic control of *N*-acetyltransferase, *Yuwiler* et al. [21] demonstrated that isoproterenol stimulation produced an increase in *N*-acetyltransferase in fetal rat pineals 2 days prior to birth. The responsiveness of fetal pineals to adrenergic stimulation was also suggested in a study by *Bäckström* et al. [5]. Although these studies suggested that β-adrenergic receptors were present before birth, the assessment of the presence of receptors depended on events which took place distal to the receptor. If, during development, constituents of the chain of events required to produce an *N*-acetyltransferase response developed at differing rates, the appearance of receptors might not be the limiting factor in the appearance of the response to adrenergic stimulation. In addition, indirect assessments of β-adrenergic receptors did not permit the quantitation of receptor number or changes

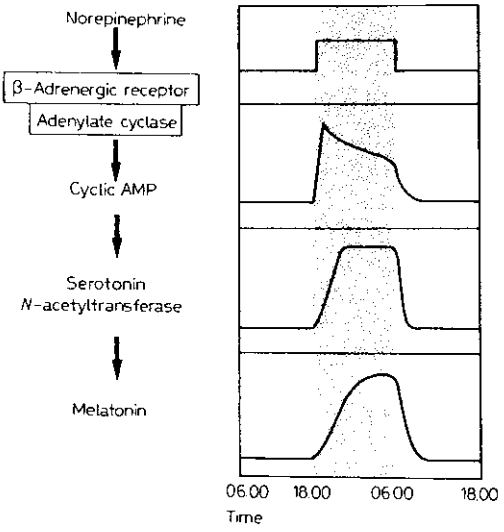

Fig. 1. Daily rhythms in the pineal gland. Data extracted from various sources [10, 11] represent changes in mature rat pineal glands. Time shows L:D 12:12 with stippled area representing dark period.

in receptor number. Even the functional assessment of receptor-mediated events was hampered in early studies by the absence of sufficiently sensitive assays or the choice of assay conditions. Thus, *Weiss* [17] reported in 1971 that pineal adenylate cyclase did not become sensitive to norepinephrine until several days after birth. The later finding that *N*-acetyltransferase activity could be stimulated by isoproterenol in fetal tissues [21] prompted a reevaluation of the appearance of adenylate cyclase activity during development.

With the availability of direct receptor binding techniques, a more precise evaluation of the time of appearance of β-adrenergic receptors and the quantitation of receptor changes was possible, independent of the effects of adrenergic stimulation on *N*-acetyltransferase activity. Additionally, better adenylate cyclase and cyclic AMP assays have permitted a reevaluation of the functional interaction of β-receptors and adenylate cyclase during the perinatal period. By focusing on these questions, specifically on changes in the perinatal period, it may be possible to better understand the functional relationships of adrenergic receptor-adenylate cyclase interactions in adult pineal tissue.

Methodology

The studies reported to date which have assessed pineal β-adrenergic receptor number by means of direct binding determinations have been carried out using the radioligands ^{3}H-dihydoalprenolol (DHA) [6, 9, 14, 18, 23] or ^{125}I-iodohydroxybenzylpindolol (IHYP) [1–3]. Both ligands are high-affinity, radiolabelled β-adrenergic antagonists which bind specifically to β-adrenergic receptors. The actual details of the binding assays vary from study to study but may be summarized in a general way as follows. Pineal tissue, either as homogenates [2, 3, 6, 9, 14, 18, 23], membrane preparations, or whole dispersed cells [1], are incubated with increasing concentrations of labelled antagonist in the presence or absence of an excess of unlabelled antagonist. Binding takes place and when it has reached equilibrium, bound counts are separated from unbound counts (free counts), usually by rapid filtration, and the concentration of bound counts is determined. High-affinity binding to β-receptors is termed specific binding. Experimentally, total binding is measured and specific binding is calculated by subtracting non-specific binding from total binding. Non-specific binding is directly measured and represents low-affinity binding to non-receptor sites which occurs in the presence a saturating concentration of unlabelled antagonist. By means of Scatchard analysis [15], i.e. examination of the relationship of specific binding to unbound ligand as the concentration of ligand increases, the maximum binding capacity and the affinity or dissociation constant of the binding ligand for the receptor are assessed.

Certain criteria must be met to establish that the observed binding is, in fact, binding to β-adrenergic receptors [20]. Among these are saturability, selectivity, and stereospecificity. DHA and IHYP behave similarly, although they differ in their affinity for the receptor. IHYP has a higher affinity and is available in higher specific activity than DHA. It lends itself to use in situations where tissue is limited. The long half-life of DHA makes it more stable but the use of a β- rather than a γ-emitter necessitates additional sample preparation. Since a number of factors influence receptor-binding including tissue preparation, binding buffer, the presence of Ca^{++} or guanine nucleotides, comparison of different studies should take these factors into account. The earliest use of DHA with pineal tissue was by *Axelrod* and co-workers [9, 14, 23] using adult rat pineals. Developmental studies were later carried out by *Greenberg and Weiss* [6] and *Weiss* et al. [18] with DHA and by *Auerbach* et al. [1–3] using IHYP.

Appearance

Using the radioligand IHYP, *Auerbach* et al. [2, 3] found that pineal β_1-adrenergic receptors are detectable in low concentrations in fetal rat pineals by 19 days gestation or 3 days prior to birth (fig. 2). This is a day earlier than was previously appreciated when *N*-acetyltransferase response was the means of indirectly assaying the presence of receptors. The fetal glands at 19 days gestation appeared to contain about 8–9 μg protein and are at the lower limit of the size feasible for use in binding studies. β-Adrenergic receptors may be present earlier in gestation but have not been measured.

Changes

During the time between 19 days gestation and 3 days of life the concentration of receptor increased about 6- to 7-fold (fig. 2, table I) [2]. By the second week of life the receptor density had more than doubled again, falling slightly by 1 month of age. The dissociation constant for binding to the receptor did not change significantly with development. In an earlier study using DHA, *Weiss* et al. [18] assessed changes in receptor density from day 1 through 2 months. They found that the most rapid postnatal receptor increase occurred between days 4 and 8, receptor density almost doubling during this time. They established and we confirmed that receptor density was greatest at the beginning of the second week of life and slowly declined thereafter. They found no developmental change in the affinity of DHA for the receptor. Although the absolute number of receptors reported by the two groups differ slightly, the postnatal increases observed were qualitatively and quantitatively similar.

Significance

The direct binding studies clearly established that there are large changes in receptor number which occur in pineal tissue prior to the development of significant sympathetic innervation [7]. They have allowed for the quantitation of the changes suggested by previous 'indirect' assessments of receptor appearance. The increase in number of receptors appears to continue during the sympathetic innervation of the gland and peaks at

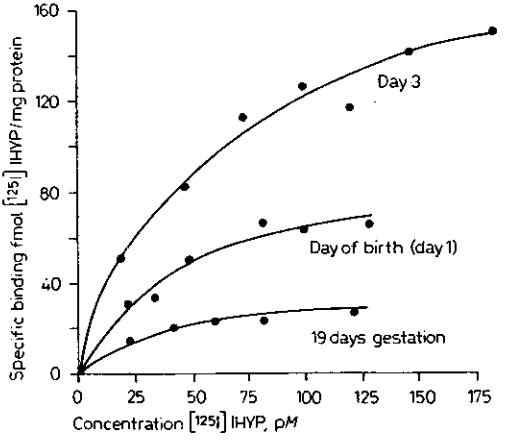

Fig. 2. ^{125}I-Iodohydroxybenzylpindolol binding to pineal gland homogenates of differing ages. Glands were collected, homogenized in buffer, and used in binding studies with increasing concentrations of IHYP [2, 3]. Specific binding is the difference between total and non-specific binding at each IHYP concentration (see text). Results are the means of triplicate determinations in representative experiments expressed as fmol bound/mg protein.

Table I. Ontogeny of β_1-adrenergic receptors in rat neural tissue: fmol of receptor/mg protein

Site	Day												Reference
	−3	1	3	4	8	10	14	21	28	42	64	90	
Cerebrum					12	18	35	74	75	75		55	13
Cerebellum					5	7	10	13	10	7		2	13
Pineal		300		350	700		725		650		650		18
Pineal	31	89	192				480		345		325		2, 3

a time which corresponds to the appearance of a diurnal melatonin rhythm [11]. In adult tissue, receptor number also undergoes a diurnal change in response to sympathetic stimulation [14]. Direct binding studies can potentially be used to determine when in development sympathetic stimulation is adequate to produce such changes.

Development in Other Neural Tissue

The appearance of β-adrenergic receptors and the changes which they undergo has been studied in other areas of rat brain. *Walton* et al. [16] demonstrated isoproterenol-responsive adenylate cyclase activity in rat cerebral cortical slices at 18 days gestation, implying the presence of β-adrenergic receptors in this tissue at this time. Direct binding studies using fetal rat cerebral cortex for quantitation of receptor number have still to be reported.

Pittman et al. [13] have used IHYP to examine the postnatal ontogeny of β-adrenergic receptors in rat cerebellum and cerebral cortex. These authors have observed that β_1-receptors in the cerebral cortex increase about 5-fold between day 8 and day 21, reaching their maximum density by the third week, about a week later than the pineal gland (table I). In the cerebellum, the overall increase in β-receptors is much less marked. Receptor number doubles during the same period seen for the cortex but only reaches about one fifth the density found in the cortex. In both of these brain areas the maximum receptor density develops later and is less than that seen in the pineal. No developmental change in receptor affinity was apparent.

As noted, the pineal studies demonstrate that large increases in receptor number occur independently of sympathetic innervation. The cortex is similar in that β-receptors can develop in the cerebral cortex independently of the presence of noradrenergic neurons [13], and even when development of neurons is blocked by 6-hydroxydopamine administration [8].

Functional Interactions

Assessment of β-adrenergic receptors during development also requires a functional correlation. Specifically, it is important to determine whether the receptors are functionally coupled to adenylate cyclase from the time they appear and whether the receptors and adenylate cyclase develop independently although they function together. To address these questions, cyclic AMP accumulation in glands in organ culture in response to both adrenergic stimulation and non-adrenergic stimulation (with cholera toxin) [12, 22] was measured.

Cyclic AMP accumulation could be stimulated by isoproterenol treatment from the time that receptors could be detected (fig. 3) and the level of accumulation appeared to reflect the receptor number present on

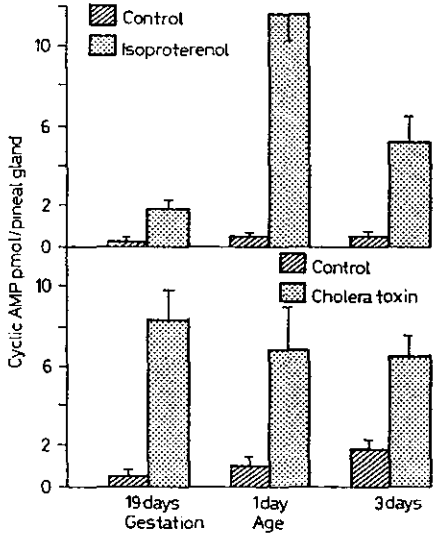

Fig. 3. Pineal cyclic AMP accumulation. Glands were collected from animals at 19 days gestation, day 1, and day 3, allowed a 1 h culture period before being stimulated with isoproterenol (10 μM, 15 min), upper panel; or cholera toxin (100 μg/ml, 1 h) lower panel. After treatment, the glands were frozen and assayed for cyclic AMP by radioimmunoassay. Cyclic AMP accumulation is expressed as pmol/pineal gland; lines above bars represent SEM [2, 3].

day 19 of gestation and day 1 of life (fig. 4) [2]. By day 3 the response appeared to have a different quantitative relationship; although receptor number was increasing, cyclic AMP accumulation had actually fallen. Thus, coupling of the receptor and adenylate cyclase appears to exist from the time the receptors appear, and significant concentrations of adenylate cyclase may be present prior to the appearance of the receptor. Investigation of the extent to which adenylate cyclase activity was present in the glands was possible using cholera toxin-activation of adenylate cyclase, an activation which is independent of a β-adrenergic receptor interaction. Cyclic AMP was measured either as it accumulated in the gland or in vitro in an assay system [3]. The accumulation of cyclic AMP in response to cholera toxin treatment on a per gland basis was remarkably similar in the glands from the three ages studied (fig. 3). Enzyme activity appeared to be similar at times when receptor numbers were dramatically different, suggesting that

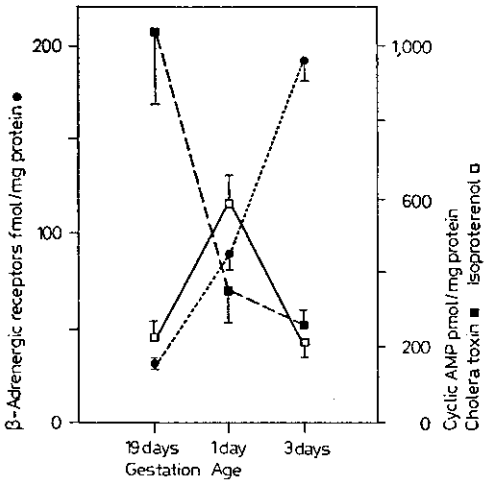

Fig. 4. Pineal β-adrenergic receptors and cyclic AMP accumulation. β-Receptors expressed as fmol/mg protein were calculated by Scatchard analysis of the binding data shown in figure 1. Cyclic AMP produced by treatment with isoproterenol (solid line) or cholera toxin (dashed line) is expressed as pmol/mg protein and reflects the data from figure 2 corrected for protein content of the glands. Note that the increase in receptor density and isoproterenol-stimulated cyclic AMP accumulation are parallel between 19 days gestation and 1 day.

early in development the level of adenylate cyclase present is regulated independently of receptor number. The lack of increased accumulation of cyclic AMP in glands from day 3 also suggested that events other than the activation of adenylate cyclase and the production of cyclic AMP might limit cyclic AMP accumulation.

Weiss [17] reported that basal levels of adenylate cyclase activity did not change from those present on day 1 but that NaF-stimulated activity rose postnatally. We have confirmed this and have extended the observation to fetal tissue (table II) [3]. When adenylate cyclase activity was measured in vitro, control values were similar at all three ages. Cholera toxin-stimulated adenylate cyclase activity appears to have increased by day 3. Although this suggested that there was more total stimulatable adenylate cyclase present at 3 days, when accumulation studies were repeated in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), no increase in cyclic AMP per mg protein was observed in glands from

Table II. Pineal adenylate cyclase activity

Age	pmol/mg protein/min
19 days gestation	
Control	54±32
Cholera toxin	253±33 *, a
Day 1	
Control	99±22
Cholera toxin	210±33 **, b
Day 3	
Control	100± 8
Cholera toxin	364±16 *, a, b

All performed in the presence of IBMX. Mean ± SEM; n = 4.
* Differs from control, p <0.01. [a] Differ from each other, p <0.01.
** Differs from control, p <0.02. [b] Differ from each other, p <0.02.

day 3 [4]. Again, this suggested that by day 3 other factors, perhaps desensitization, changes in precursor pool size within the gland, or exposure to light, modulate the expression of adenylate cyclase activity even if the basic functional coupling of the β-receptor and the adenylate cyclase is unchanged.

Conclusion (table III)

β-Adrenergic receptors are detectable in fetal rat pineal glands 3 days prior to birth. These receptors increase in number dramatically during the perinatal period, reach peak density by the end of the second week of life and decline in number thereafter. Pineal β-adrenergic receptors develop prior to the establishment of significant functional sympathetic innervation of the gland. Pineal β-receptors increase earlier and reach a higher density than β-receptors in rat cerebral cortex or cerebellum.

β-Adrenergic receptors in the pineal appear to be functionally coupled to adenylate cyclase from the time they can be detected in direct binding studies. Earlier in development, receptor number and adenylate cyclase activity, though related functionally, appear to be regulated somewhat independently. Later in development, both receptor number and adenylate cyclase activity are additionally modulated by sympathetic stimulation.

Table III. Development of adrenergic control of gene expression in the rat pineal gland

Index	Time
Serotonin *N*-acetyltransferase	
Appearance	1 day prior to birth
Rhythmic activity	end of first week
Sympathetic innervation	begins at 2 days
β-Adrenergic receptors	
Appearance	by 19 days gestation
Early changes	7-fold increase by 3 days
Adenylate cyclase	
Appearance	before 19 days gestation
Functional coupling	as receptors appear, modified by sympathetic innervation

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